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(57) Abstract

A process for producing a small–sized, lipid–based cochleate is described. Cochleates are derived from liposomes which are suspended in an aqueous two–phase polymer solution, enabling the differential partitioning of polar molecule based–structures by phase separation. The liposome–containing two–phase polymer solution, treated with positively charged molecules such as Ca^{2+} or Zn^{2+}, forms a cochleate precipitate of a particle size less than one micron. The process may be used to produce cochleates containing biologically relevant molecules. Small–sized cochleates may be administered orally or through the mucosa to obtain an effective method of treatment.

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NOVEL HYDROGEL ISOLATED COCHLEATE FORMULATIONS, PROCESS OF PREPARATION AND THEIR USE FOR THE DELIVERY OF BIOLOGICALLY RELEVANT MOLECULES

RELEVANT MOLECULES

FIELD OF THE INVENTION

The present invention relates to a novel process for preparing a novel lipid-based cochleate delivery system, the pharmaceutical preparations derived from the lipid-based cochleate delivery system, and the use of these pharmaceutical preparations to achieve efficient systemic and mucosal delivery of biologically relevant molecules.

BACKGROUND OF THE INVENTION

The ability of biologically relevant molecules to be administered via the oral route depends on several factors. The biologically relevant molecule must be soluble in the gastrointestinal fluids in order for the biologically relevant molecule to be transported across biological membranes for an active transport mechanism, or have suitable small particle size that can be absorbed through the Peyer's Patches in the small intestine and through the lymphatic system. Particle size is an important parameter when oral delivery is to be achieved (see Couvreur P. et al, Adv. Drug Delivery Reviews 10:141-162, 1993).

The primary issue in the ability to deliver drugs orally is the protection of the drug from proteolytic enzymes. An ideal approach is to incorporate the drug in a hydrophobic material so that the aqueous fluids cannot penetrate the system. Lipid-based cochleates are an ideal system that can achieve this purpose.

The advantages of cochleates are numerous. The cochleates have a nonaqueous structure and therefore they:

a) are more stable because of less oxidation of lipids;

b) can be stored lyophilized, which provides the potential to be stored for long periods of time at room temperatures, which would be advantageous for worldwide shipping and storage prior to administration;

c) maintain their structure even after lyophilization, whereas liposome structures are destroyed by lyophilization;

d) exhibit efficient incorporation of biologically relevant molecules into the lipid bilayer of the cochleate structure;

e) have the potential for slow release of a biologically relevant molecule *in vivo* as cochleates dissociate;

f) have a lipid bilayer which serves as a carrier and is composed of simple lipids which are found in animal and plant cell membranes, so that the lipids are non-toxic;

g) are produced easily and safely;

h) can be produced as defined formulations composed of predetermined amounts and ratios of drugs or antigens.

Cochleate structures have been prepared first by D. Papahadjopoulos as an intermediate in the preparation of large unilamellar vesicles (see US 4,078,052). The use of cochleates to deliver protein or peptide molecules for vaccines has been disclosed in US 5,840,707. However, neither of these patents addresses the importance of particle size or the effective oral delivery of biologically relevant molecules mediated by small-sized cochleates.

SUMMARY OF THE INVENTION

Accordingly, it is an object of this invention to provide a method for obtaining a hydrogel-isolated cochleate of a particle size less than one micron. The method further comprises the steps required to encochleate at least one biologically relevant molecule in the hydrogel-isolated cochleates in a therapeutically effective amount.

A "biologically relevant molecule" is one that has a role in the life processes of a living organism. The molecule may be organic or inorganic, a monomer or a polymer, endogenous to a host organism or not, naturally occurring or synthesized in vitro, and the like. Thus, examples include vitamins, minerals, amino acids, toxins, microbicides, microbistats, co-factors, enzymes, polypeptides, polypeptide aggregates, polynucleotides, lipids, carbohydrates, nucleotides, starches, pigments, fatty acids, hormones, cytokines, viruses, organelles, steroids and other multi-ring structures, saccharides, metals, metabolic poisons, drugs, and the like.

These and other objects have been obtained by providing an encochleated biologically relevant molecule, wherein the biologically relevant molecule-cochleate comprises the following components:

a) a biologically relevant molecule,

b) a negatively charged lipid, and

c) a cation component,

wherein the particle size of the encochleated biologically relevant molecule is less than one micron, and further wherein the biologically relevant molecule is preferably a drug.

The present invention further provides a method of orally administering to a host a biologically effective amount of the above-described cochleate.

In a preferred embodiment, the biologically relevant molecule-cochleate is administered orally.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Schematic of the process by which the hydrogel-isolated cochleates with or without drug are obtained.

Figures 2a and 2b. Particle size distribution (weight analysis) of hydrogel isolated cochleates either loaded with amphotericin B (AmB) (fig 2a) or empty (fig 2b) as measured by laser light scattering.

Figures 3a and 3b. Microscopic images of a mixture of liposomes in dextran dispersed into PEG gel solution. The small black dots are dextran particles formed by dispersing the dextran phase in the PEG phase. The large open circles are formed by fusion of small dextran particles. Partition of liposomes favors the dextran phase as indicated by the yellow color of AmB. 3b. Microscopic images of the sample shown in Fig. 3a after treatment with $CaCl_2$ solution. The black objects in circles, indicated by an arrow, are cochleates formed by the addition of Ca^{2+} ions.

Figures 4a-4f. Microscopic images of the sample shown in Figs. 3a and 3b after washing with a buffer containing 1 mM $CaCl_2$ and 100 mM NaCl. Aggregates are formed by the Cochleate particles. 4b. Suspension shown in Fig. 4a following the addition of EDTA. Cochleate particles opened to liposomes with a diameter of 1-2 microns, indicating the intrinsic size of the cochleate particles is in sub-micron range. 4c. AmB hydrogel isolated-cochleates precipitated with zinc according to the procedure described in Example 4. 4d. Cochleates

displayed in fig 4c after treatment with EDTA. 4e. Empty hydrogel isolated-cochleates precipitated with zinc according to the procedure described in Example 3. 4f) cochleates displayed in 4f after treatment with EDTA.

Figure 5. Micrographs of hydrogel-isolated cochleates after freeze fracture.

Figure 6. Growth inhibition of Candida albicans by hydrogel-isolated cochleates loaded with AmB at 0.625 μg AmB/ml. Comparison is made to AmB in DMSO and AmBisome^R.

Figure 7. Effect of hydrogel-isolated cochleates on the viability of Candida albicans after 30 hours.

Figure 8. Efficacy of Amphotericin B-cochleates and macrophage cultures.

Figure 9. Amphotericin B tissue levels after administration of Amphotericin B-cochleates

Figure 10. Time profile tissue concentration of AmB after single dose administration of hydrogel-isolated cochleates loaded with AmB.

Figure 11. AmB tissue level 24 hrs after single dose and 24 hrs after a multiple dose regime.

Figure 12. Correlation between Amphotericin B tissue level and the level of Candida albicans after administration of Amphotericin B cochleates.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a solution to achieve effective oral delivery of drugs by producing small-sized cochleates using a new process. The new approach is based on the incompatibility between two polymer solutions, both of which are aqueous. Aqueous two-phase systems of polymers are well used for protein purification due to a number of advantages such as freedom from the need for organic solvents, mild surface tension and the biocompatibility of aqueous polymers (see P.A. Albertsson in "Partition of cell particles and

macromolecules", 3rd edition, Wiley NY 1986; and "Separation using aqueous Phase System" D. Fisher Eds, Plenum NY, 1989). It is known, for example, that large polar molecules such as proteins partition to a much higher concentration in a polymer phase with the physical characteristics similar to those of dextran than in a polymer phase with the physical characteristics similar to those of PEG (D. Forciniti, C. K. Hall, M. R. Kula, Biotechnol. Bioeng. 38, 986 1991).

According to the present invention there are provided processes for preparing small-sized, lipid-based cochleate particles and preparations derived therefrom, comprising a biologically relevant molecule incorporated into the particles. The cochleate particles are formed of an alternating sequence of lipid bilayers/cation. The biologically relevant molecule is incorporated either in the lipid bilayers or in the interspace between the lipid bilayers. One of the processes for preparing the small-sized cochleates comprises: 1) preparing a suspension of small unilamellar liposomes or biologically relevant molecule-loaded liposomes, 2) mixing the liposome suspension with polymer A, 3) adding, preferably by injection, the liposome/Polymer A suspension into another polymer B in which polymer A is nonmiscible, leading to an aqueous two-phase system of polymers, 4) adding a solution of cation salt to the two-phase system of step 3, such that the cation diffuses into polymer B and then into the particles comprised of liposome/polymer A allowing the formation of small-sized cochleates, 5) washing the polymers out and resuspending the empty or drug-loaded cochleates into a physiological buffer or any appropriate pharmaceutical vehicle.

A second process for preparing the small-sized cochleates comprises detergent and a biologically relevant molecule and cation. The process comprises the following steps:

a) providing an aqueous suspension containing a detergent-lipid mixture;

b) mixing the detergent-lipid suspension with polymer A;

c) adding the detergent-lipid/polymer A suspension into a solution comprising polymer B, wherein polymer A and polymer B are immiscible, thereby creating a two-phase polymer system;

d) adding a solution of a cationic moiety to the two-phase polymer system; and

e) washing the two-phase polymer system to remove the polymer.

A lyophilization procedure can be applied and the lyophilized biologically relevant molecule-cochleate complex can be filled into soft or hard gelatin capsules, tablets or other dosage form, for systemic, dermal or mucosal delivery.

This process leads to a small-sized particle with a narrow size range that allows efficient oral delivery of biologically relevant molecules. The biologically relevant molecule partitions into either or both lipid bilayers and interspace, and the biologically relevant molecule is released from the cochleate particles by dissociation of the particles *in vivo*. Alternative routes of administration may be systemic such as intramuscular, subcutaneous or intravenous or mucosal such as intranasal, intraocular, intravaginal, intraanal, parenteral or intrapulmonary. Appropriate dosages are determinable by, for example, dose-response experiments in laboratory animals or in clinical trials and taking into account body weight of the patient, absorption rate, half-life, disease severity and the like. The number of doses, daily dosage and course of treatment may vary from individual to individual. Other delivery routes can be dermal, transdermal or intradermal.

The first step of the new process of the present invention, which is the preparation of small liposomes, can be achieved by standard methods such as sonication or microfluidization or other related methods (see for example Liposome Technology, Liposome

Preparation and Related Techniques, Edited by Gregory Gregoriadis, Vol I, 2nd Edition, CRC Press, 1993).

The addition, preferably by injection, of polymer A/liposome suspension into polymer B can be achieved mechanically by using a syringe pump at an appropriate controlled rate, for example a rate of 0.1 ml/min to 50 ml/min and preferably at a rate of 1 to 10 ml/min.

The lipids of the present invention are non-toxic lipids and include, but are not limited to simple lipids which are found in animal and plant cell membranes. Preferably the lipid is a negatively charged lipid, more preferably a negatively charged phospholipid, and even more preferably a lipid from the group of phosphatidylserine, phosphatidylinositol, phosphatidic acid, and phosphatidyl glycerol. The lipids may also include minor amounts of zwitterionic lipids, cationic lipids or neutral lipids capable of forming hydrogen bonds to a biologically relevant molecule such as PEGylated lipid.

The polymers A and B of the present invention can be of any biocompatible polymer classes that can produce an aqueous two-phase system. For example, polymer A can be, but is not limited to, dextran 200,000-500,000, Polyethylene glycol (PEG) 3,400-8,000; polymer B can be, but is not limited to, polyvinylpyrrolidone (PVP), polyvinylalcohol (PVA), Ficoll 30,000-50,000, polyvinyl methyl ether (PVMB) 60,000-160,000, PEG 3,400-8,000. The concentration of polymer A can range from between 2-20% w/w as the final concentration depending on the nature of the polymer. The same concentration range can be applied for polymer B. Examples of suitable two-phase systems are Dextran/PEG, 5-20% w/w Dextran 200,000-500,000 in 4-10% w/w PEG 3,400-8,000; Dextran/PVP 10-20% w/w Dextran 200,000-500,000 in 10-20% w/w PVP 10,000-20,000; Dextran/PVA 3-15% w/w Dextran 200,000-500,000 in 3-15% w/w PVA 10,000-60,000; Dextran/Ficoll 10-20% w/w Dextran

200,000-500,000 in 10-20% w/w Ficoll 30,000-50,000; PEG/PVME 2-10% w/w PEG 3,500-35,000 in 6-15% w/w PVME 60,000-160,000.

The biologically relevant molecule may be an organic molecule that is hydrophobic in aqueous media. The biologically relevant molecule may be a drug, and the drug may be an antiviral, an anesthetic, an anti-infectious, an antifungal, an anticancer, an immunosuppressant, a steroidal anti-inflammatory, a non-steroidal anti-inflammatory, a tranquilizer or a vasodilatory agent. Examples include Amphotericin B, acyclovir, adriamycin, cabamazepine, melphalan, nifedipine, indomethacin, naproxen, estrogens, testosterones, steroids, phenytoin, ergotamines, cannabinoids rapamycin, propanidid, propofol, alphadione, echinomycine, miconazole nitrate, teniposide, taxol, and taxotere.

The biologically relevant molecule may be a polypeptide such as cyclosporin, angiotensin I, II and III, enkephalins and their analogs, ACTH, anti-inflammatory peptides I, II, III, bradykinin, calcitonin, b-endorphin, dinorphin, leucokinin, leutinizing hormone releasing hormone (LHRH), insulin, neurokinins, somatostatin, substance P, thyroid releasing hormone (TRH) and vasopressin.

The biologically relevant molecule may be an antigen, but the antigen is not limited to a protein antigen. The antigen can also be a carbohydrate or a polynucleotide such as DNA. Examples of antigenic proteins include envelope glycoproteins from influenza or Sendai viruses, animal cell membrane proteins, plant cell membrane proteins, bacterial membrane proteins and parasitic membrane protein.

The biologically relevant molecule is extracted from the source particle, cell, tissue, or organism by known methods. Biological activity of biologically relevant molecules need not be maintained. However, in some instances (e.g., where a protein has membrane fusion or

ligand binding activity or a complex conformation which is recognized by the immune system), it is desirable to maintain the biological activity. In these instances, an extraction buffer containing a detergent which does not destroy the biological activity of the membrane protein is used. Suitable detergents include ionic detergents such as cholate salts, deoxycholate salts and the like or heterogeneous polyoxyethylene detergents such as Tween, BRIG or Triton.

Utilization of this method allows reconstitution of antigens, more specifically proteins, into the liposomes with retention of biological activities, and eventually efficient association with the cochleates. This avoids organic solvents, sonication, or extreme pH, temperature, or pressure all of which may have an adverse effect upon efficient reconstitution of the antigen in a biologically active form.

Hydrogel-isolated cochleates may contain a combination of various biologically relevant molecules as appropriate.

The formation of small-sized cochleates (with or without a biologically relevant molecule) is achieved by adding a positively charged molecule to the aqueous two-phase polymer solution containing liposomes. In the above procedure for making cochleates, the positively charged molecule can be a polyvalent cation and more specifically, any divalent cation that can induce the formation of a cochleate. In a preferred embodiment, the divalent cations include Ca^{++}, Zn^{++}, Ba^{++} and Mg^{++} or other elements capable of forming divalent ions or other structures having multiple positive charges capable of chelating and bridging negatively charged lipids. Addition of positively charged molecules to liposome-containing solutions is also used to precipitate cochleates from the aqueous solution.

To isolate the cochleate structures and to remove the polymer solution, cochleate precipitates are repeatedly washed with a buffer containing a positively charged molecule, and more preferably, a divalent cation. Addition of a positively charged molecule to the wash buffer ensures that the cochleate structures are maintained throughout the wash step, and that they remain as precipitates.

The medium in which the cochleates are suspended can contain salt such as calcium chloride, zinc chloride, cobalt chloride, sodium chloride, sodium sulfate, potassium sulfate, ammonium sulfate, magnesium sulfate and sodium carbonate. The medium can contain polymers such as Tween 80 or BRIG or Triton. The biologically relevant molecule-cochleate is made by diluting into an appropriate biologically acceptable carrier (e.g., a divalent cation-containing buffer).

The cochleate particles can be enteric. The cochleate particles can be placed within gelatin capsules and the capsule can be enteric coated.

In the preparations of the present invention certain hydrophobic materials can be added to provide enhanced absorption properties for oral delivery of biologically relevant molecules. These materials are preferably selected from the group consisting of long chain carboxylic acids, long chain carboxylic acid esters, long chain carboxylic acid alcohols and mixtures thereof. The hydrophobic materials can be added either initially to the lipid prior to the formation of liposomes or in a later step in the form of a fat vehicle such as an emulsion.

The skilled artisan can determine the most efficacious and therapeutic means for effecting treatment practicing the instant invention. Reference can also be made to any of numerous authorities and references including, for example, "Goodman & Gillman's, The

Pharmaceutical Basis for Therapeutics", (6th Ed., Goodman et al., eds., MacMillan Publ. Co., New York, 1980).

The invention will now be described by examples which are not to be considered as limiting the invention. In the examples, unless otherwise indicated, all ratios, percents and amounts are by weight.

EXAMPLES

Example 1. Preparation of empty hydrogel-isolated cochleates from dioleoylphosphatidylserine precipitated with calcium

Step 1 : Preparation of small unilamellar vesicles from dioleoylphosphatidylserine.

A solution of dioleoyl phosphatidylserine (DOPS, Avanti Polar Lipids, Alabaster, AL, USA) in chloroform (10 mg/ml) was placed in a round-bottom flask and dried to a film using a Buchi rotavapor at 35 °C. The rotavapor was sterilized by flashing nitrogen gas through a 0.2 μm filter. The following steps were carried out in a sterile hood. The dried lipid film was hydrated with de-ionized water at the concentration of 10 mg lipid/ml. The hydrated suspension was purged and sealed with nitrogen, then sonicated in a cooled bath sonicator (Laboratory Supplies Com., Inc.). Sonication was continued (for several seconds to several minutes depending on lipid quantity and nature) until the suspension became clear (suspension A) and there were no liposomes apparently visible under a phase contrast microscope with a 1000X magnification. Laser light scattering (weight analysis, Coulter N4 Plus) indicates that the mean diameter is 35.7 ± 49.7 nm.

Step 2 : Preparation of hydrogel isolated cochleates

The liposome suspension obtained in step 1 was then mixed with 40 % w/w dextran-500,000 (Sigma) in a suspension of 2/1 v/v Dextran/liposome. This mixture was then

injected with a syringe into 15 % w/w PEG-8,000 (Sigma) [PEG 8000/(suspension A)] under magnetic stirring to result in suspension B. The rate of the stirring was 800-1,000 rpm. A $CaCl_2$ solution (100 mM) was added to the suspension to reach the final concentration of 1 mM.

Stirring was continued for one hour, then a washing buffer containing 1 mM $CaCl_2$ and 150 mM NaCl was added to suspension B at the volumetric ratio of 1:1. The suspension was vortexed and centrifuged at 3000 rpm, 2-4 °C, for 30 min. After the supernatant was removed, additional washing buffer was added at the volumetric ratio of 0.5:1, followed by centrifugation under the same conditions. A schematic of this new process of obtaining cochleates is detailed in Figure 1. The resultant pellet was reconstituted with the same buffer to the desired concentration. Laser light scattering (weight analysis, Coulter N4 Plus) indicates that the mean diameter for the cochleate is 407.2 ± 85 nm (figure 2b).

Example 2. Preparation of empty hydrogel-isolated cochleates from a mixture of dioleoylphosphatidylserine and 1,2-Distearoyl-sn-glycerol-3-phosphoethanolamine-n-(poly(ethylene glycol)-5000, DSPE-PEG) precipitated with calcium

Step 1 : Preparation of small unilamellar vesicles.

A solution of dioleoylphosphatidylserine (DOPS) and 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-n-(poly(ethylene glycol)-5000), (DSPE-PEG, Avanti Polar Lipids, Alabaster, AL, USA) in chloroform (ratio of DOPS:DSPS-PEG = 100:1, w:w) was placed in a round-bottom flask and dried to a film using a Buchi rotavapor at 35 °C. The rotavapor was sterilized by flashing nitrogen gas through a 0.2 μm filter. The following steps were carried out in a sterile hood. The dried lipid film was hydrated with de-ionized water to a concentration of 10 mg lipid/ml. The hydrated suspension was purged and sealed with nitrogen, then sonicated in a cooled bath sonicator (Laboratory Supplies Com., Inc.).

Sonication was continued (for several seconds to several minutes depending on lipid quantity and nature) until the suspension became clear (suspension A) and there were no liposomes apparently visible under a phase contrast optical microscope with a 1000X magnification.

Step 2 : Preparation of hydrogel isolated cochleates

The liposome suspension obtained in step 1 was then mixed with 40 % w/w dextran-500,000 in a suspension of 2/1 v/v Dextran/liposome. This mixture was then injected *via* a syringe into 15 % w/w PEG-8,000 [PEG 8000/(suspension A)] under magnetic stirring to result in suspension B. The rate of the stirring was 800-1,000 rpm. A $CaCl_2$ solution (100 mM) was added to the suspension to reach the final concentration of 1 mM.

Stirring was continued for one hour, then a washing buffer containing 1 mM $CaCl_2$ and 150 mM NaCl was added to suspension B at the volumetric ratio of 1:1. The suspension was vortexed and centrifuged at 3000 rpm, 2-4 °C, for 30 min. After the supernatant was removed, additional washing buffer was added at the volumetric ratio of 0.5:1, followed by centrifugation under the same conditions. A schematic of this new process of obtaining cochleates is detailed in Figure 1. The resulting pellet was reconstituted with the same buffer to the desired concentration. Phase contrast optical microscopy indicates the formation of uniform, very small, needle-like cochleates.

Example 3. Preparation of empty hydrogel-isolated cochleates from a mixture of dioleoylphosphatidylserine and n-octyl-beta-D-gluco-pyranoside precipitated with calcium

Step 1 : Preparation of small unilamellar vesicles.

A solution of dioleoylphosphatidylserine (DOPS) in chloroform was placed in a round-bottom flask and dried to a film using a Buchi rotavapor at 35 °C. The rotavapor was sterilized by flashing nitrogen gas through a 0.2 μm filter. The following steps were carried

out in a sterile hood. The dried lipid film was hydrated with a solution of n-octyl-beta-D-gluco-pyranoside (OCG) at 1 mg/ml at a ratio of DOPS : OCG of 10:1 w:w. The hydrated suspension was purged and sealed with nitrogen, then sonicated briefly in a cooled bath sonicator.

Step 2 : Preparation of hydrogel isolated cochleates

The suspension obtained in step 1 was then mixed with 40 % w/w dextran-500,000 in a suspension of 2/1 v/v Dextran/liposome. This mixture was then injected *via* a syringe into 15 % w/w PEG-8,000 [PEG 8000/(suspension A)] under magnetic stirring to result in suspension B. The rate of the stirring was 800-1,000 rpm. A $CaCl_2$ solution (100 mM) was added to the suspension to reach the final concentration of 1 mM.

Stirring was continued for one hour, then a washing buffer containing 1 mM $CaCl_2$ and 150 mM NaCl was added to suspension B at the volumetric ratio of 1:1. The suspension was vortexed and centrifuged at 3000 rpm, 2-4 °C, for 30 min. After the supernatant was removed, additional washing buffer was added at the volumetric ratio of 0.5:1, followed by centrifugation under the same conditions. A schematic of this new process of obtaining cochleates is detailed in Figure 1. The resulting pellet was reconstituted with the same buffer to the desired concentration. Phase contrast optical microscopy indicates the formation of uniform, very small, needle-like cochleates.

Example 4. Preparation of Amphotericin B-loaded hydrogel-isolated cochleates precipitated with calcium

Step 1: Preparation of small unilamellar AmB-loaded, vesicles from dioleoylphosphatidylserine.

A mixture of dioleoyl phosphatidylserine (DOPS) in chloroform (10 mg/ml) and AmB in methanol (0.5mg/ml) at a molar ratio of 10:1 was placed in a round-bottom flask and

dried to a film using a Buchi rotavapor at 40 °C. The rotavapor was sterilized by flashing nitrogen gas through a 0.2 μm filter. The following steps were carried out in a sterile hood. The dried lipid film was hydrated with de-ionized water at the concentration of 10 mg lipid/ml. The hydrated suspension was purged and sealed with nitrogen, then sonicated in a cooled bath sonicator. Sonication was continued (for several seconds to several minutes depending on lipid quantity and nature) until the suspension became clear yellow (suspension A) and there were no liposomes apparently visible under a phase contrast microscope with a 1000X magnification.

Step 2 : Preparation of AmB-loaded, hydrogel- isolated cochleates

The liposome suspension obtained in step 1 was then mixed with 40 % w/w dextran-500,000 in a suspension of 2/1 v/v Dextran/liposome. This mixture was then injected *via* a syringe into 15 % w/w PEG-8,000 [PEG 8000/(suspension A)] under magnetic stirring to result in suspension B. The rate of the stirring was 800-1,000 rpm. A $CaCl_2$ solution (100 mM) was added to the suspension to reach the final concentration of 1mM.

Stirring was continued for one hour, then a washing buffer containing 1 mM $CaCl_2$ and 150 mM NaCl was added to suspension B at the volumetric ratio of 1:1. The suspension was vortexed and centrifuged at 3000 rpm, 2-4 °C, for 30 min. After the supernatant was removed, additional washing buffer was added at the volumetric ratio of 0.5:1, followed by centrifugation under the same conditions. A schematic of this new process of obtaining cochleates is detailed in Figure 1. The resulting pellet was reconstituted with the same buffer to the desired concentration. Laser light scattering (weight analysis, Coulter N4 Plus) indicate that the AmB-cochleates mean diameter was 407.3 $\pm$ 233.8 nm (figure 2a).

Example 5. Preparation of Doxorubicin (DXR) loaded hydrogel isolated cochleates precipitated with calcium

Step :1 Preparation of small unilamellar DXR-loaded vesicles from dioleoylphosphatidylserine.

A mixture of dioleoylphosphatidylserine (DOPS) in chloroform (10 mg/ml) and (DXR) in methanol (0.5mg/ml) at a molar ratio of 10:1 was placed in a round-bottom flask and dried to a film using a Buchi rotavapor at room temperature. The rotavapor was sterilized by flashing nitrogen gas through a 0.2 μm filter. The following steps were carried out in a sterile hood. The dried lipid film was hydrated with de-ionized water at the concentration of 25 mg lipid/ml. The hydrated suspension was purged and sealed with nitrogen, then sonicated in a cooled bath sonicator. Sonication was continued (for several seconds to several minutes depending on lipid quantity and nature) until the suspension became clear pink (suspension A) and there were no liposomes apparently visible under phase contrast microscope with a 1000X magnification.

Step 2 : Preparation of DXR-loaded, hydrogel isolated cochleates

5 ml of the liposome suspension obtained in step 1 was then mixed with 40 % w/w dextran-500,000 (Sigma) in a suspension of 2/1 v/v Dextran/liposome. This mixture was then injected *via* a syringe into 15 % w/w PEG-8,000 [PEG 8000/(suspension A)] under magnetic stirring to result in suspension B. The rate of the stirring was 800-1,000 rpm. A $CaCl_2$ solution (100 mM) was added to the suspension to reach the final concentration of 1mM.

Stirring was continued for one hour, then a washing buffer containing 1 mM $CaCl_2$ and 150 mM NaCl was added to suspension B at the volumetric ratio of 1:1. The suspension was vortexed and centrifuged at 6400 rpm, 2-4 °C, for 30 min. A schematic of this new

process of obtaining cochleates is detailed in Figure 1. The resulting pellet was reconstituted with the same buffer to the desired concentration. Laser light scattering (weight analysis, Coulter N4 Plus) confirmed the formation of small DXR-cochleates.

Example 6. Preparation of Cyclosporin A (CSPA) -loaded hydrogel isolated cochleates precipitated with calcium

Step 1 : Preparation of small unilamellar CSPA-loaded vesicles from dioleoylphosphatidylserine.

A mixture of dioleoylphosphatidylserine (DOPS) in chloroform (10 mg/ml) and CSPA in methanol (0.5mg/ml) at a molar ratio of 10:1 was placed in a round-bottom flask and dried to a film using a Buchi rotavapor at room temperature. The rotavapor was sterilized by flashing nitrogen gas through a 0.2 μm filter. The following steps were carried out in a sterile hood. The dried lipid film was hydrated with de-ionized water at the concentration of 10 mg lipid/ml. The hydrated suspension was purged and sealed with nitrogen, then sonicated in a cooled bath sonicator. Sonication was continued (for several seconds to several minutes depending on lipid quantity and nature) until the suspension became clear (suspension A) and there were no liposomes apparently visible under a phase contrast microscope with a 1000X magnification.

Step 2 : Preparation of CSPA-loaded, hydrogel isolated, cochleates

The liposome suspension obtained in step 1 was then mixed with 40 % w/w dextran-500,000 in a suspension of 2/1 v/v Dextran/liposome. This mixture was then injected *via* a syringe into 15 % w/w PEG-8,000 (Sigma) [PEG 8000/(suspension A)] under magnetic stirring to result in suspension B. The rate of the stirring was 800-1,000 rpm. A $CaCl_2$ solution (100 mM) was added to the suspension to reach the final concentration of 1mM.

Stirring was continued for one hour, then a washing buffer containing 1 mM $CaCl_2$ and 150 mM NaCl was added to suspension B at the volumetric ratio of 1:1. The suspension was vortexed and centrifuged at 3000 rpm, 2-4 °C, for 30 min. After the supernatant was removed, additional washing buffer was added at the volumetric ratio of 0.5:1, followed by centrifugation under the same conditions. A schematic of this new process of obtaining cochleates is detailed in Figure 1. The resulting pellet was reconstituted with the same buffer to the desired concentration. Laser light scattering (weight analysis, Coulter N4 Plus) confirmed the formation of small CSPA-cochleates.

Example 7. Preparation of Nelfinavir (NVIR)loaded hydrogel isolated cochleates precipitated with calcium

Step 1 : Preparation of small unilamellar NVIR-loaded vesicles from dioleoylphosphatidylserine.

A mixture of dioleoylphosphatidylserine (DOPS) in chloroform (10 mg/ml) and NVIR in methanol (0.5mg/ml) at a molar ratio of 10:1 was placed in a round-bottom flask and dried to a film using a Buchi rotavapor at RT. The rotavapor was sterilized by flashing nitrogen gas through a 0.2 μm filter. The following steps were carried out in a sterile hood. The dried lipid film was hydrated with de-ionized water at the concentration of 10 mg lipid/ml. The hydrated suspension was purged and sealed with nitrogen, then sonicated in a cooled bath sonicator. Sonication was continued (for several seconds to several minutes depending on lipid quantity and nature) until the suspension became clear (suspension A) and there were no liposomes apparently visible under a phase contrast microscope with a 1000X magnification.

Step 2 : Preparation of NVIR-loaded, hydrogel isolated, cochleates

The liposome suspension obtained in step 1 was then mixed with 40 % w/w dextran-500,000 in a suspension of 2/1 v/v Dextran/liposome. This mixture was then injected *via* a syringe into 15 % w/w PEG-8,000 [PEG 8000/(suspension A)] under magnetic stirring to result in suspension B. The rate of the stirring was 800-1,000 rpm. A $CaCl_2$ solution (100 mM) was added to the suspension to reach the final concentration of 1mM.

Stirring was continued for one hour, then a washing buffer containing 1 mM $CaCl_2$ and 150 mM NaCl was added to suspension B at the volumetric ratio of 1:1. The suspension was vortexed and centrifuged at 3000 rpm, 2-4 °C, for 30 min. After the supernatant was removed, additional washing buffer was added at the volumetric ratio of 0.5:1, followed by centrifugation under the same conditions. A schematic of this new process of obtaining cochleates is detailed in Figure 1. The resulting pellet was reconstituted with the same buffer to the desired concentration. Laser light scattering (weight analysis, Coulter N4 Plus) confirmed the formation of small NVIR-cochleates.

Example 8. Preparation of Rifampin (RIF) -loaded hydrogel-isolated cochleates precipitated with calcium

Step 1 : Preparation of small unilamellar RIF-loaded vesicles from dioleoylphosphatidylserine.

A mixture of dioleoylphosphatidylserine (DOPS) in chloroform (10 mg/ml) and RIF in methanol (0.5mg/ml) at a molar ratio of 10:1 was placed in a round-bottom flask and dried to a film using a Buchi rotavapor at RT. The rotavapor was sterilized by flashing nitrogen gas through a 0.2 μm filter. The following steps were carried out in a sterile hood. The dried lipid film was hydrated with de-ionized water at the concentration of 10 mg lipid/ml. The hydrated suspension was purged and sealed with nitrogen, then sonicated in a cooled bath

sonicator. Sonication was continued (for several seconds to several minutes depending on lipid quantity and nature) until the suspension became clear (suspension A) and there were no liposomes apparently visible under a phase contrast microscope with a 1000X magnification.

Step 2 : Preparation of RIF-loaded, hydrogel isolate,d cochleates

The liposome suspension obtained in step 1 was then mixed with 40 % w/w dextran-500,000 (Sigma) in a suspension of 2/1 v/v Dextran/liposome. This mixture was then injected *via* a syringe into 15 % w/w PEG-8,000 (Sigma) [PEG 8000/(suspension A)] under magnetic stirring to result in suspension B. The rate of the stirring was 800-1,000 rpm. A $CaCl_2$ solution (100 mM) was added to the suspension to reach the final concentration of 1mM.

Stirring was continued for one hour, then a washing buffer containing 1 mM $CaCl_2$ and 150 mM NaCl was added to suspension B at the volumetric ratio of 1:1. The suspension was vortexed and centrifuged at 3000 rpm, 2-4 °C, for 30 min. After the supernatant was removed, additional washing buffer was added at the volumetric ratio of 0.5:1, followed by centrifugation under the same conditions. A schematic of this new process of obtaining cochleates is detailed in Figure 1. The resulting pellet was reconstituted with the same buffer to the desired concentration. Laser light scattering (weight analysis, Coulter N4 Plus) confirmed the formation of small RIF-cochleates.

Example 9. Preparation of Vitamin A loaded hydrogel isolated cochleates precipitated with calcium

Step 1 : Preparation of small unilamellar Vitamin A-loaded vesicles from dioleoylphosphatidylserine.

Vitamin A (retinol) is sensitive to air oxidation and is inactivated by UV light. Vitamin A is protected when embedded into lipid bilayers. The incorporation is achieved as follows :

A mixture of dioleoylphosphatidylserine (DOPS) in chloroform (10 mg/ml) and Vitamin A in methanol (0.5mg/ml) at a molar ratio of 10:1 was placed in a round-bottom flask and dried to a film using a Buchi rotavapor at RT. The rotavapor was sterilized by flashing nitrogen gas through a 0.2 μm filter. The following steps were carried out in a sterile hood. The dried lipid film was hydrated with de-ionized water at the concentration of 10 mg lipid/ml. The hydrated suspension was purged and sealed with nitrogen, then sonicated in a cooled bath sonicator. Sonication was continued (for several seconds to several minutes depending on lipid quantity and nature) until the suspension became clear (suspension A) and there were no liposomes apparently visible under a phase contrast microscope with a 1000X magnification.

Step 2 : Preparation of Vitamin A-loaded, hydrogel isolated, cochleates

The liposome suspension obtained in step 1 was then mixed with 40 % w/w dextran-500,000 in a suspension of 2/1 v/v Dextran/liposome. This mixture was then injected *via* a syringe into 15 % w/w PEG-8,000 [PEG 8000/(suspension A)] under magnetic stirring to result in suspension B. The rate of the stirring was 800-1,000 rpm. A $CaCl_2$ solution (100 mM) was added to the suspension to reach the final concentration of 1mM.

Stirring was continued for one hour, then a washing buffer containing 1 mM $CaCl_2$ and 150 mM NaCl was added to suspension B at the volumetric ratio of 1:1. The suspension was vortexed and centrifuged at 3000 rpm, 2-4 °C, for 30 min. After the supernatant was removed, additional washing buffer was added at the volumetric ratio of 0.5:1, followed by centrifugation under the same conditions. A schematic of this new process of obtaining cochleates is detailed in Figure 1. The resulting pellet was reconstituted with the same buffer to the desired concentration.

Example 10. Preparation of polyunsaturated fatty acid (PFA) loaded hydrogel isolated cochleates precipitated with calcium

PFA's are biologically relevant molecules involved in the control of the level of cholesterol in blood and are the precursors of prostaglandins. PFA's are sensitive to oxidation which limits their incorporation in food. PFA's undergo, in the presence of oxygen, a series of reactions called autoxidation, leading to aldehydes then ketones which has a fishy unpleasaant odor and flavor. Embedding PFA in rigid, enrolled, lipid bilayers help preventing the autoxidation cascade. A general method of preparing PFA- cochleates is as follows :

Step 1 : Preparation of small unilamellar PFA-loaded vesicles from dioleoylphosphatidylserine.

A mixture of dioleoylphosphatidylserine in chloroform (10 mg/ml) and PFA in methanol (0.5mg/ml) at a molar ratio of 10:1 was placed in a round-bottom flask and dried to a film using a rotary evaporator at RT. The rotary evaporator was sterilized by flashing nitrogen gas through a 0.2 μm filter. The following steps were carried out in a sterile hood. The dried lipid film was hydrated with de-ionized water at the concentration of 10 mg lipid/ml. The hydrated suspension was purged and sealed with nitrogen, then sonicated in a cooled bath sonicator. Sonication was continued (for several seconds to several minutes

depending on lipid quantity and nature) until the suspension became clear (suspension A) and there were no liposomes apparently visible under a phase contrast microscope with a 1000X magnification.

Step 2 : Preparation of PFA-loaded, hydrogel isolated, cochleates

The liposome suspension obtained in step 1 was then mixed with 40 % w/w dextran-500,000 in a suspension of 2/1 v/v Dextran/liposome. This mixture was then injected *via* a syringe into 15 % w/w PEG-8,000 [PEG 8000/(suspension A)] under magnetic stirring to result in suspension B. The rate of the stirring was 800-1,000 rpm. A $CaCl_2$ solution (100 mM) was added to the suspension to reach the final concentration of 1mM.

Stirring was continued for one hour, then a washing buffer containing 1 mM $CaCl_2$ and 150 mM NaCl was added to suspension B at the volumetric ratio of 1:1. The suspension was vortexed and centrifuged at 3000 rpm, 2-4 °C, for 30 min. After the supernatant was removed, additional washing buffer was added at the volumetric ratio of 0.5:1, followed by centrifugation under the same conditions. A schematic of this new process of obtaining cochleates is detailed in Figure 1. The resulting pellet was reconstituted with the same buffer to the desired concentration.

Example 11. Preparation of Cinnamon Oil (CinO) loaded hydrogel isolated cochleates precipitated with calcium

Step 1 : Preparation of small unilamellar CinO-loaded vesicles from dioleoylphosphatidylserine.

A mixture of dioleoylphosphatidyl serine (DOPS) in chloroform (10 mg/ml) and CinO in methanol (0.5mg/ml) at a molar ratio of 10:1 was placed in a round-bottom flask and dried to a film using a Buchi rotavapor at 40 °C. The rotavapor was sterilized by flashing nitrogen gas through a 0.2 μm filter. The following steps were carried out in a sterile hood.

The dried lipid film was hydrated with de-ionized water at the concentration of 10 mg lipid/ml. The hydrated suspension was purged and sealed with nitrogen, then sonicated in a cooled bath sonicator. Sonication was continued (for several seconds to several minutes depending on lipid quantity and nature) until the suspension became clear (suspension A) and there were no liposomes apparently visible under a phase contrast microscope with a 1000X magnification.

Step 2 : Preparation of CinO-loaded, hydrogel isolated, cochleates

The liposome suspension obtained in step 1 was then mixed with 40 % w/w dextran-500,000 in a suspension of 2/1 v/v Dextran/liposome. This mixture was then injected *via* a syringe into 15 % w/w PEG-8,000 [PEG 8000/(suspension A)] under magnetic stirring to result in suspension B. The rate of the stirring was 800-1,000 rpm. A $CaCl_2$ solution (100 mM) was added to the suspension to reach the final concentration of 1mM.

Stirring was continued for one hour, then a washing buffer containing 1 mM $CaCl_2$ and 150 mM NaCl was added to suspension B at the volumetric ratio of 1:1. The suspension was vortexed and centrifuged at 3000 rpm, 2-4 °C, for 30 min. After the supernatant was removed, additional washing buffer was added at the volumetric ratio of 0.5:1, followed by centrifugation under the same conditions. A schematic of this new process of obtaining cochleates is detailed in Figure 1. The resulting pellet was reconstituted with the same buffer to the desired concentration.

Example 12. Preparation of DNA loaded hydrogel isolated cochleates precipitated with calcium

Step 1 : Preparation of small unilamellar DNA-loaded vesicles from dioleoylphosphatidylserine.

A solution of dioleoylphosphatidylserine in chloroform (10 mg/ml) was placed in a round-bottom flask and dried to a film using a Buchi rotavapor at RT. The rotavapor was sterilized by flashing nitrogen gas through a 0.2 μm filter. The following steps were carried out in a sterile hood. The dried lipid film was hydrated with a solution of pCMV-beta-gal-DNA in TE buffer (at 1 mg/ml) to reach a concentration of DOPS:DNA of 10:1 and a concentration of 10 mg lipid/ml. The hydrated suspension was purged and sealed with nitrogen, then vortexed for several minutes.

Step 2 : Preparation of DNA-loaded, hydrogel isolated, cochleates

The DNA/liposome mixture was then mixed with 40 % w/w dextran-500,000 in a suspension of 2/1 v/v Dextran/liposome. This mixture was then injected *via* a syringe into 15 % w/w PEG-8,000 [PEG 8000/(suspension A)] under magnetic stirring to result in suspension B. The rate of the stirring was 800-1,000 rpm. A $CaCl_2$ solution (100 mM) was added to the suspension to reach the final concentration of 1mM.

Stirring was continued for one hour, then a washing buffer containing 1 mM $CaCl_2$ and 150 mM NaCl was added to suspension B at the volumetric ratio of 10:1. The suspension was vortexed and centrifuged at 3000 rpm, 2-4 °C, for 30 min. After the supernatant was removed, additional washing buffer was added at the volumetric ratio of 5:1, followed by centrifugation under the same conditions. A schematic of this new process of

obtaining cochleates is detailed in Figure 1. The resulting pellet was reconstituted with the same buffer to the desired concentration.

Example 13. Preparation of empty hydrogel isolated-cochleates precipitated with zinc

Step 1 : Preparation of small unilamellar vesicles from dioleoylphosphatidylserine.

A solution of dioleoylphosphatidylserine (DOPS) in chloroform (10 mg/ml) was placed in a round-bottom flask and dried to a film using a Buchi rotavapor at 35 °C. The rotavapor was sterilized by flashing nitrogen gas through a 0.2 μm filter. The following steps were carried out in a sterile hood. The dried lipid film was hydrated with de-ionized water at the concentration of 10 mg lipid/ml. The hydrated suspension was purged and sealed with nitrogen, then sonicated in a cooled bath sonicator. Sonication was continued (for several seconds to several minutes depending on lipid quantity and nature) until the suspension became clear (suspension A) and there were no liposomes apparently visible under a phase contrast microscope with a 1000X magnification.

Step 2 : Preparation of hydrogel isolated cochleates

The liposome suspension obtained in step 1 was then mixed with 40 % w/w dextran-500,000 in a suspension of 2/1 v/v Dextran/liposome. This mixture was then injected *via* a syringe into 15 % w/w PEG-8,000 [PEG 8000/(suspension A)] under magnetic stirring to result in suspension B. The rate of the stirring was 800-1,000 rpm. A $ZnCl_2$ solution (100 mM) was added to the suspension to reach the final concentration of 1 mM.

Stirring was continued for one hour, then a washing buffer containing 1 mM $ZnCl_2$ and 150 mM NaCl was added to suspension B at the volumetric ratio of 1:1. The suspension was vortexed and centrifuged at 3000 rpm, 2-4 °C, for 30 min. After the supernatant was

removed, additional washing buffer was added at the volumetric ratio of 0.5:1, followed by centrifugation under the same conditions. A schematic of this new process of obtaining cochleates is detailed in Figure 1. The resulting pellet was reconstituted with the same buffer to the desired concentration. Laser light scattering (weight analysis, Coulter N4 Plus) confirmed the formation of small cochleates.

Example 14. Preparation of Amphotericin B-loaded hydrogel-isolated cochleates precipitated with zinc

Step 1 : Preparation of small unilamellar AmB-loaded vesicles from dioleoylphosphatidylserine.

A mixture of dioleoyl phosphatidylserine (DOPS) in chloroform (10 mg/ml) and AmB in methanol (0.5 mg/ml) at a molar ratio of 10:1 was placed in a round-bottom flask and dried to a film using a Buchi rotavapor at 40 °C. The rotavapor was sterilized by flashing nitrogen gas through a 0.2 μm filter. The following steps were carried out in a sterile hood. The dried lipid film was hydrated with de-ionized water at the concentration of 10 mg lipid/ml. The hydrated suspension was purged and sealed with nitrogen, then sonicated in a cooled bath sonicator. Sonication was continued (for several seconds to several minutes depending on lipid quantity and nature) until the suspension became clear yellow (suspension A) and there were no liposomes apparently visible under a phase contrast microscope with a 1000X magnification.

Step 2 : Preparation of AmB-loaded, hydrogel isolated cochleates

The liposome suspension obtained in step 1 was then mixed with 40 % w/w dextran-500,000 in a suspension of 2/1 v/v Dextran/liposome. This mixture was then injected *via* a syringe into 15 % w/w PEG-8,000 [PEG 8000/(suspension A)] under magnetic stirring to

result in suspension B. The rate of the stirring was 800-1,000 rpm. A $ZnCl_2$ solution (100 mM) was added to suspension to reach the final concentration of 1 mM.

Stirring was continued for one hour, then a washing buffer containing 1 mM $ZnCl_2$ and 150 mM NaCl was added to the suspension B at the volumetric ratio of 1:1. The suspension was vortexed and centrifuged at 3000 rpm, 2-4 °C, for 30 min. After the supernatant was removed, additional washing buffer was added at the volumetric ratio of 0.5:1, followed by centrifugation under the same conditions. A schematic of this new process of obtaining cochleates is detailed in Figure 1. The resultant pellet was reconstituted with the same buffer to the desired concentration. Laser light scattering (weight analysis, Coulter N4 Plus) confirmed the formation of small AmB-Zn-cochleates.

Example 15. Microscopic observation of hydrogel-isolated cochleates

Optical microscopic study was performed stepwise alone with the preparation procedure in order to gain some mechanistic details of the hydrogel-isolated cochleates formation.

The microscopic images seen in Figures 3a,b and 4a-f show the morphological changes at each preparation step of AmB loaded hydrogel isolated-cochleates precipitated with Ca^{2+} ions. When the AmB/liposome-dextran mixture was dispersed into PEG solution, phase separation resulted as shown by Fig. 3a. Partition of the liposomes favored the dispersed dextran phase as indicated by a yellow color of AmB. This partitioning ensures that liposomes are isolated in each dextran particles. Addition of Calcium ions into the continued phase (PEG) resulted in formation of precipitates in the dispersed phase. As the final product, small needle-shape cochleates were formed and observed under the microscope, these cochleates opened into unilamellar vesicles upon addition of EDTA and

chelation of the calcium (Figures 4a,b). The needle-shaped morphology was confirmed by scanning electron microscopy after freeze-fracture (Figure 5). Similar microscopic images were obtained for empty and AmB-Zn-precipitated hydrogel isolated-cochleates (Figures 4c,d) and empty Zn-precipitated hydrogel isolated-cochleate (Figures 4e,f).

Example 16. Antifungal Activity of hydrogel isolated cochleates loaded with Amphotericin B, *in vitro*

Growth Inhibition of Candida albicans

An *in vitro* yeast susceptibility assay was performed comparing the inhibitory and lethal effects of AmB-cochleates, AmBisomes (liposomal formulation of AmB) and AmB/DMSO. Five colonies of freshly growing *Candida albicans* were selected from a YPD agar plate (from a 48 hour culture) and added to 2 ml of 2x YPD broth, pH 5.7. The OD_{590} of this stock culture was measured and the yeast density was adjusted to $OD_{590} = 0.1$ and 0.1ml of this suspension added to each well of a 96 well plate.

AmB/cochleates, AmB/DMSO and AmBisomes were added to 96 well plates to a final concentration of 0.078, 0.156, 0.3125, 0.625, 1.25 and 2.5 μg/ml of AmB. The 96 well plates were incubated at 37°C with gentle shaking and cell density was measured on a 96 well plate reader (Molecular Devices Spectramax 340) at 0, 2, 4, 6, 24 and 30 hours. Figure 6 shows that AmB-cochleates have a greater growth inhibitory effect than AmBisomes (liposomal formulation of AmB).

Fungicidal effect of hydrogel isolated cochleates loaded with Amphotericin B.

Aliquots of yeast cells (50 μl) were removed from the 96 well plates and serially diluted (up to 1:10000 for plating onto agar plates) and counted using a hemocytometer. Fifty μl of the diluted yeast cells were plated onto YPD agar plates and incubated for 24

hours at 37°C. Yeast colonies were counted using a BioRad Fluor-S Multi-Imager equipped with Quanitity One™ software.

Yeast cells treated with AmBisome, AmB/DMSO and AmB/cochleates (0.625 ug AmB/ml) were examined for the ratio of colony forming units to total cell number after 30 hours of incubation. The results show that the AmB/cochleates had the greatest lethal effect on the yeast cells compared to the other antifungal agents tested. There was nearly 0% yeast viability after treatment with the AmB-cochleates and 12% yeast viability after treatment with AmB/DMSO. The AmBisome was not as effective, resulting in 52% yeast viability (Figure 7).

Macrophage Protection with AmB cochleates.

Particle scavenging cells, such as macrophage, are the first line of defense against many microbial infections. However, many microbes, which induce severe human clinical infections, have been shown to infect macrophage and avoid destruction.

It is possible that *in vivo,* macrophage play an important role in the uptake of cochleates, *via* an endocytotic mechanism. Since macrophage also play an important role in the host defense and clearance of fungi and parasites, it is important to study the interaction between macrophage and cochleates.

The following examples indicate that the cochleates are taken up by macrophage. Large doses of AmB delivered to the macrophage were found to be non-toxic and remained within the macrophage in a biologically active form. AmB cochleates provided protection for the macrophage against infection by *Candida albicans* when administered prior to or after fungal infection.

Prophylactic dose regime: J774A.1 macrophage (M) were subcultured into a 96-well plate at a concentration of $1x10^5$ cells/ml in DMEM +10% FBS. 100µl AmB cochleates (AmBc 0.2, 0.6, 1.25, and 2.5 µg AmB/ml), Fungizone, or empty cochleates (EC at 2, 6, 12.5, and 25µg lipid/ml) were added at the specified concentration. Plates were incubated overnight at 37°C and 5% CO_2. 24 hours later, the medium was replaced. This step was performed twice. *Candida albicans* (CA) was added to the plate at a concentration of $2.5x10^3$ cells/ml, a ratio of 1:200 with respect to the macrophage. Plates were incubated overnight under the conditions stated above.

Following the 24 hr incubation, the plates were removed and observed. Medium was pipetted vigorously to remove and disrupt the cells, 25µl of this suspension was placed onto Sabouraud Dextrose Agar plates, and then placed in a dry incubator overnight at 37°C. *C. albicans* CFU's were counted the following day. The data in Figure 8 suggest that AmB cochleate loaded macrophage are very effective at killing the fungal cells.

Post-infection dose regime: J774A.1 macrophage (M) were subcultured into a 96-well plate then incubated overnight. Following incubation, the macrophage were infected with CA at a ratio of 200:1, then subsequently AmBc, Fungizone or EC was added at the specified concentrations. Twenty four hours later, the cell cultures were observed and CFU's determined as described above.

When M were challenged with CA and subsequently dosed with AmB cochleates, the CFU count was again nearly zero. These results indicate that macrophage engulf and concentrate AmB cochleate, as macrophage were protected against *C. albicans* challenge after AmB cochleate had been washed off (Figure 8).

In contrast, Fungizone, (AmB in deoxycholate), the most popular clinical form of AmB was extremely toxic and lethal to the macrophage *in vitro*. Within 5 hours of administration, there was a large amount of cellular debris found in the petri dish, with no signs of viable macrophage.

Microscopic observation reveals the AmB cochleates are not toxic to the macrophage even at the highest doses studied. The AmB cochleates are accumulated at high levels resulting in large distended vacuoles. After washing of the macrophage and incubating again for 24 hr, most of the vacuoles had returned to the normal shape and size, yet a few were noticeably enlarged. A few macrophage were even noticed to be "moving" with the enlarged vacuoles. AmB cochleates are concentrated within the vacuoles and it is probable that AmB is released gradually over time.

Example 17 : Evaluation of tissue penetration of AmB after iv administration of amphotericin b hydrogel isolated cochleates

Tissue penetration of amphotericin B has been evaluated after iv administration. Groups (n=5) of C57BL/6 mice (20 –23 g) were given iv (0.625 mg/kg) AmB cochleates (0.05 ml/ 20 g) with a ½ cc U 100 insulin syringe with a 18 g ½ needle size. At predetermined sacrifice times (2, 5, 10, 20 and 40 min, 1, 2, 3, 4, 6, 8, 12, 24, 36 and 48 hrs), animals were giving anesthesia, blood collected via cardiac puncture, then euthanized, dissected and tissues of interest removed (brain, lung, liver, spleen, kidneys, heart, fat, stomach, stomach contents, intestine and intestine contents) and weighed. For analysis of AmB, samples were mixed with extraction solvent (10% methanol, 35% water, 55% ethanol), homogenized, sonicated and centrifuged. A 90 µl aliquot of supernatant was transfered into a micro vial, injected into the HPLC system in a Nova-Pak C-18 column (3.9x150 mm, 4 µm particle size) kept at 40 °C. Amphotericin B was eluted at a flow rate of 0.5 ml/min with 29%

methanol, 30% acetonitrile and 41% 2.5 mM EDTA and detected at 408 nm. The concentration of AmB was calculated with the help of an external standard curve.

In Figure 9 the tissue exposure after a single iv dose of AmB cochleates is shown. Large penetration in key tissues like liver, spleen and kidney can be observed.

Example 18 : Oral delivery of AmB mediated by hydrogel isolated cochleates loaded with AmB.

Single dose regime

Oral availability of the hydrogel isolated cochleates loaded with AmB has been examined by intragastric administration of the formulation of example 2 to overnight fasting, C57BL16 mice (20-23g). One tenth ml of the formulation at the dose of 10 mg/kg was administrated to 9 mice. Three mice from each group were sacrificed at 1, 6 and 24 hrs post administration followed by analysis of AmB level in organs and tissues.

Tissue and blood samples were processed as follows: tissues were diluted 1/20 or 1/10 by addition of extraction solvent (H_2O 35%, methanol 10%, ethanol 55% w/w/w nv/v/v) and homogenized with an Ultra-Turrex® device. A 0.5 ml aliquot was taken, sonicated for 1 min and centrifuged at 7260 rpm for 12 min at 4°C. Supernatant was transferred to an HPLC micro-vial and 30 µl injected on a C-18, 3.9 x 150 mm, 4 µm particle sized analytical column with a flow rate of 0.5 ml, at 40°C. Concentration of AmB detected at 408 nm was calculated with the help of an external calibration curve.

Figure 10 shows the time profile of AmB in the tissues over a period f time of 24 hrs. Although only three time points are plotted, accumulation in key tissues (liver, lungs, spleen and kidneys) can be seen.

Multiple dose regime

Two other groups of mice received a 10 mg/kg/day oral multiple dose regime for ten days and one group was sacrificed 24 hrs after the last dose and the other group 20 days after the last dose received. At the predetermined time points mice were anesthetized, sacrificed and dissected for tissue collection. Tissues were processed as in single dose regime and AmB level determined by HPLC. Results from 24 h after the 10^{th} dose are depicted in Figure 11 and show that hydrogel isolated cochleates allow the delivery of AmB from the gastrointestinal tract at therapeutic levels.

Example 19 : Correlation between biodistribution in healthy and infected mice and the level of *Candida albicans* in tissue after oral administration

Figure 12 shows the relationship between tissue levels of amphotericin B (μg/g tissue on left scale) and efficacy as decrease of *Candida albicans* infection (CFU/g on the right scale) after oral administration of AmB-cochleates (Figure 12).

After oral administration of 10 mg/kg/day for 10 consecutive days to healthy mice, AmB presented high levels in kidneys followed by lungs, spleen, liver and brain, which shows much lower levels than the other tissues. It has been shown that disease state affects pharmacokinetics of drugs at different levels. This phenomenon can be seen clearly in the graph: AmB in tissue reaches lower levels in *C. albicans* infected mice after oral administration of 10 mg/kg/day (same dose) for 15 days, 5 more doses than the healthy group. It also shows a change in the distribution pattern where the lungs are the target tissue with lowest levels.

Oral administration of an AmB cochleate formulation at 10 mg/kg/day for 15 days provided high efficacy. The decrease in CFU/g in kidney tissue is about 3.5 logs for the cochleate formulation. In lungs, AmB cochleate formulations completely eradicate *C.*

albicans and clears the lungs of fungal infection. It is clear that the cochleate delivery system provides a high level of AmB in infected animals, this correlates with the higher efficiency seen in the cochleate formulation, indicating that AmB-cochleates are a suitable vehicle for oral treatment of systemic Candidiasis.

In addition, orally administered AmB-cochleates were non-toxic even at the highest dose of 50 mg/kg (no lesions were found in kidneys, GI tract and other organs of mice given 10, 20 and 50 mg/kg of AmB-cochleates). This high dose (50mg/kg) is equivalent to 100 times the lowest dose (0.5mg/kg) that showed 100% of survival in the *Candida* infected mouse model.

What is Claimed:

1. A method for producing lipid-based cochleates comprising the steps of:

a) providing an aqueous suspension of liposomes;

b) mixing the liposome suspension with polymer A;

c) adding the liposome/polymer A suspension into a solution comprising polymer B, wherein polymer A and polymer B are immiscible, thereby creating a two-phase polymer system;

d) adding a solution of a cationic moiety to the two-phase polymer system; and

e) washing the two-phase polymer system to remove the polymers.

2. A method of claim 1 wherein the addition of liposome/polymer A suspension is done by injection.

3. A method of claim 1 or claim 2 wherein the liposomes are formed of lipid comprising negatively charged lipid.

4. A method of claim 3 wherein the negatively charged lipid is phosphatidylserine.

5. A method of claim 3 or claim 4 wherein the lipid comprises a minor amount of other lipids.

6. A method of claim 5 wherein the other lipid is selected from the group of zwitterionic lipids.

7. A method of claim 5 wherein the other lipid is selected from the group of cationic lipids.

8. A method of claim 5 wherein the other lipids are selected from the group of lipids capable of forming hydrogen bonds to a biologically active molecule.

9. A method of claim 8 wherein the neutral lipid is a PEGylated lipid.

10. A method of any preceding claim wherein polymer A is at least one member selected from the group consisting of dextran and polyethylene glycol.

11. A method of any preceding claim wherein the concentration of polymer A in the two-phase polymer system ranges in concentration from 2 to 20% w/w.

12. A method of any preceding claim wherein polymer B is at least one member selected from the group consisting of polyvinylpyrrolidone, polyvinylalcohol, Ficoll, polyvinyl methyl ether, and polyethylene glycol.

13. A method of any preceding claim wherein the concentration of polymer B in the two-phase polymer system is the range of 2 to 20% w/w.

14. A method of any preceding claim wherein the two-phase polymer solution is at least one member selected from the group consisting of dextran/polyethylene glycol, dextran/polyvinylpyrrolidone, dextran/polyvinylalcohol, dextran/Ficoll and polyethylene glycol/polyvinyl methyl ether.

15. A method of any preceding claim wherein the cationic moiety comprises a di- or higher- valent ion.

16. A method of any preceding claim wherein the cationic moiety comprises a di- or higher- valent metal ion.

17. A method of claim 16 wherein the metal ion is added as a salt of an inorganic acid, preferably wherein the salt is calcium chloride, zinc chloride or cobalt chloride.

18. A method of any preceding claim wherein the lipid cochleates are small-sized cochleates.

19. A method according to claim 18 wherein the size of the cochleates is less than 1 micron.

20. A method according to claim 18 wherein the cochleates are formed using small unilamellar liposomes.

21. A method according to any preceding claim wherein the aqueous suspension of liposomes comprises a biologically relevant molecule, whereby the cochleates also comprise the biologically relevant molecule.

22. A method according to claim 21 which comprises the preliminary step of forming the liposome suspension by forming a film comprising a mixture of the biologically relevant molecule and lipid and contacting the film with an aqueous phase to form the aqueous suspension of small unilamellar liposomes.

23. A method according to claim 21 in which the biologically relevant molecule is present in the aqueous phase.

24. A method according to claim 23 comprising the preliminary step of forming liposomes in which a film of lipid is contacted with aqueous phase containing the biologically relevant molecule to form the aqueous suspension of liposomes.

25. A method according to claim 21 including a preliminary step in which a suspension of empty liposomes is mixed with the biologically relevant molecule to form the said liposome suspension.

26. A method according to claim 23 wherein the biologically relevant molecule bears a charge.

27. A method according to claim 26 wherein the biologically relevant molecule is positively charged.

28. A method according to claim 26 wherein the biologically relevant molecule is negatively charged.

29. A method according to claim 23 wherein the negatively charged molecule is a polynucleotide.

30. A method according to claim 29 wherein the polynucleotide is DNA.

31. A method for producing lipid-based cochleates comprising the steps of:

a) providing an aqueous suspension containing a detergent-lipid mixture;

b) mixing the detergent-lipid suspension with polymer A;

c) adding the detergent-lipid/polymer A suspension into a solution comprising polymer B, wherein polymer A and polymer B are immiscible, thereby creating a two-phase polymer system;

d) adding a solution of a cationic moiety to the two-phase polymer system; and

e) washing the two-phase polymer system to remove the polymer.

32. A method for producing lipid-based cochleates according to claim 31 wherein the detergent is octyl glucoside.

33. A method for producing lipid-based cochleates according to claim 31 wherein the cochleate comprises a biologically active molecule.

34. A method according to claim 33 wherein the biologically active molecule bear a charge.

35. A method according to claim 33 wherein the biologically active molecule is positively charged.

36. A method according to claim 33 wherein the biologically active molecule is negatively charged.

37. A method according to claim 36 wherein the negatively charged molecule is a polynucleotide.

38. A method according to claim 37 wherein the polynucleotide is DNA.

39. A method according to claim 31 wherein the biologically active molecule is added in step a.

40. A method according to claim 21 or claim 33 in which the biologically relevant molecule is selected from the group consisting of drugs, polynucleotides, polypeptides and antigens.

41. A method of claim 40 wherein the biologically relevant molecule is a drug selected from the group consisting of antiviral agents, anesthetics, antibacterial agents, antifungal agents, anticancer agents, immunosuppressants, steroidal anti-inflammatory agents, non-steroidal anti-inflammatory agents, tranquilisers and vasodilators.

42. A method of claim 41 in which the drug is at least one member selected from the group consisting of Amphotericin B, acyclovir, adriamycin, cabamazepine, melphalan, nifedipine, indomethacin, naproxen, estrogens, testosterones, steroids, phenytoin, ergotamines, cannabinoids rapamycin, propanidid, propofol, alphadione, echinomycin, miconazole nitrate, teniposide, taxol and taxotere.

43. A method according to any preceding claim in which the washing step involved centrifuging the two-phase polymer system to separate the cochleate precipitate, removing the supernatant containing the polymer, resuspending the precipitate in a washing buffer, centrifuging the washed precipitate , and optionally repeating the resuspension and centrifugation steps one or more times.

44. A method according to claim 43 in which the washing buffer contains dissolved cationic moiety.

45. A method according to claim 44 in which the cationic moiety comprises di- or higher- valent ions.

46. A method according to claim 45 in which the di- or higher- valent ions are metal ions.

47. A method according to claim 46 in which the metal ions are selected from calcium and zinc.

48. A method according to any of claims 43 to 47 in which the cationic moiety is present in the washing buffer at a concentration of at least 1mM.

49. Cochleates containing a biologically relevant molecule prepared according to claims 1 to 48.

50. Cochleates containing a biologically relevant molecule according to claim 49 having a mean particle size less than 1 micron.

51. Cochleates according to claim 49 wherein the biologically relevant molecules are selected from the group consisting of drugs, polynucleotides, polypeptides and antigens.

52. Cochleates according to claim 51 wherein the biologically relevant molecule is a drug selected from the group consisting of antiviral agents, anesthetics, antibacterial agents, antifungal agents, anticancer agents, immunosuppressants, steroidal anti-inflammatory agents, non-steriodal anti-inflammatory agents, tranquilisers and vasodilators.

53. Cochleates according to claim 52 wherein the drug is at least one member selected from the group consisting of Amphotericin B, acyclovir, adriamycin, cabamazepine, melphalan, nifedipine, indomethacin, naproxen, estrogens, testosterones, steroids, phenytoin, ergotamines, cannabinoids rapamycin, propanidid, propofol, alphadione, echinomycin, miconazole nitrate, teniposide, taxol and taxotere.

54. Cochleates according to any of claims 49 to 53, or the product of a method of any of claims 1 to 48, for use in a method of delivering a biologically relevant molecule to a host.

55. Use of cochleates according to any of claims 49 to 54, or of the product of a method according to claims 1 to 48, in the manufacture of a composition for use in the method of treatment of the human or animal body or to deliver a biologically relevant molecule to a host.

56. A method of treatment comprising administering to a human or animal cochleates according to any of claims 49 to 54, or the product of any of claims 1 to 48.

57. Use according to claim 55 or method according to claim 56 in which the administration is by a mucosal or a systemic route.

58. Use or method of claim 57 wherein the administration is a mucosal route selected from oral, intranasal, intraocular, intraanal, intravaginal, parenteral or intrapulmonary.

59. Use or method according to claim 57 in which the route is oral.

60. Use or method according to claim 58 in which the administration is proceeded by aerosol.

61. Use or method according to claim 57 wherein the administration is systemic and is by a route selected from intravenous, intramuscular, subcutaneous, transdermal or intradermal.

62. A composition comprising cochleates according to any of claims 49 to 54 of the product of the process according to any of claims 1 to 48, and a carrier.

63. A pharmaceutical composition comprising cochleates according to any of claims 49 to 54 or the product of a process according to any of claims 1 to 48, and a pharmaceutically acceptable carrier.

Figure 1

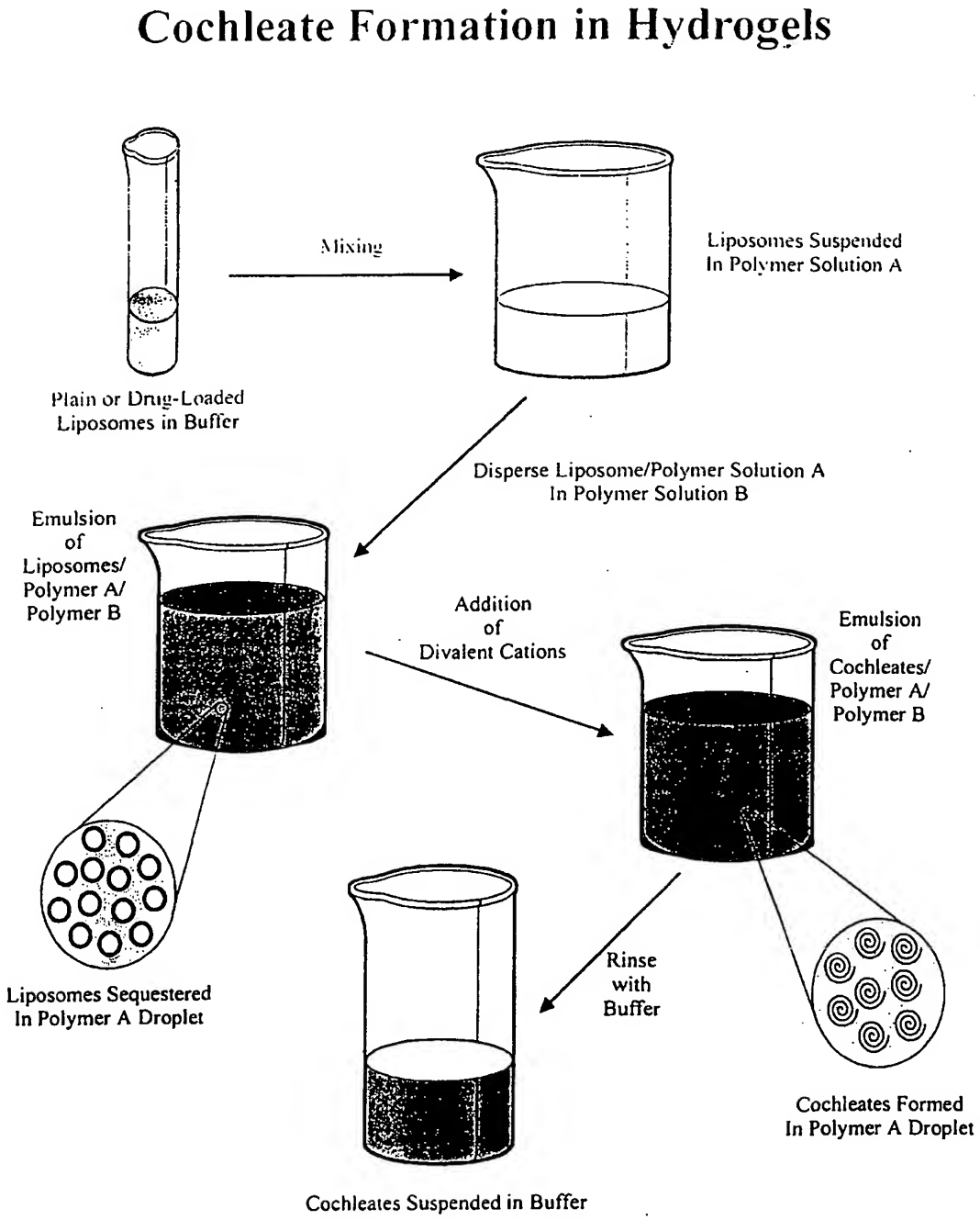
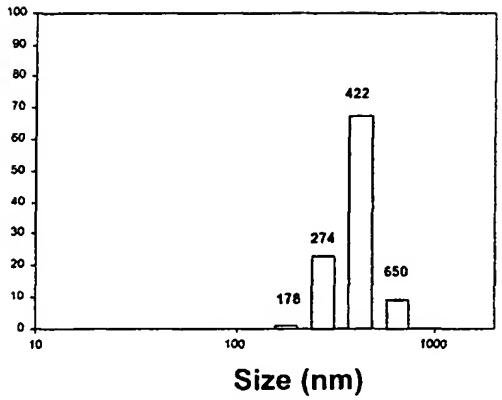

Cochleate Formation in Hydrogels
Mixing
Liposomes Suspended
In Polymer Solution A
Plain or Drug-Loaded
Liposomes in Buffer
Disperse Liposome/Polymer Solution A
In Polymer Solution B
Emulsion
of
Liposomes/
Polymer A/
Polymer B
Addition
of
Divalent Cations
Emulsion
of
Cochleates/
Polymer A/
Polymer B
Liposomes Sequestered
In Polymer A Droplet
Rinse
with
Buffer
Cochleates Formed
In Polymer A Droplet
Cochleates Suspended in Buffer

Figure 2

a


100
90
80
70
60
50
40
30
20
10
0
422
274
650
178
10
100
1000
Size (nm)

b

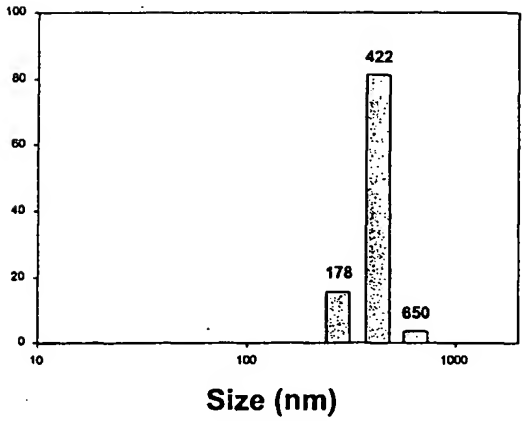
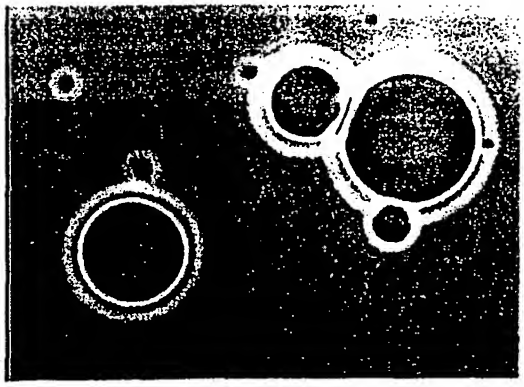

100
80
60
40
20
0
422
178
650
10
100
1000
Size (nm)

Figure 3

a



b

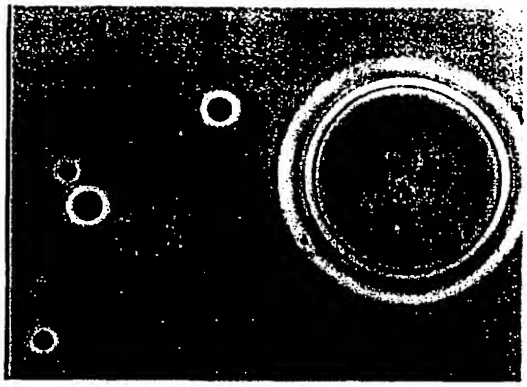
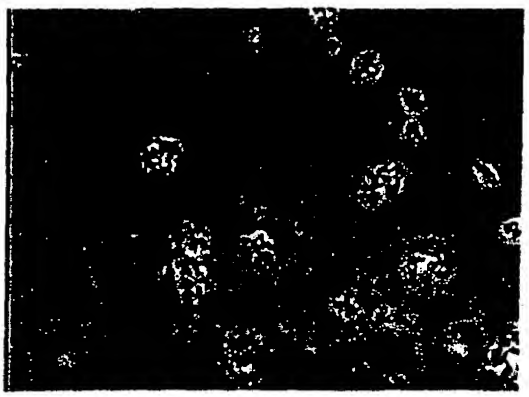
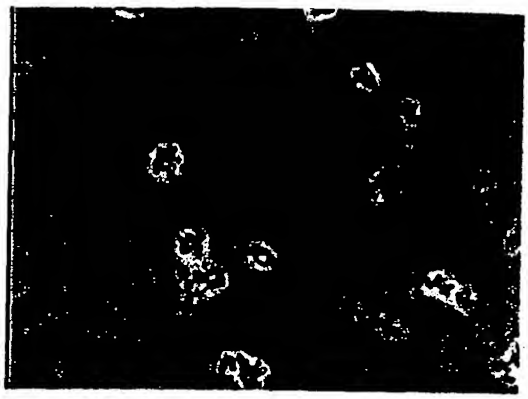


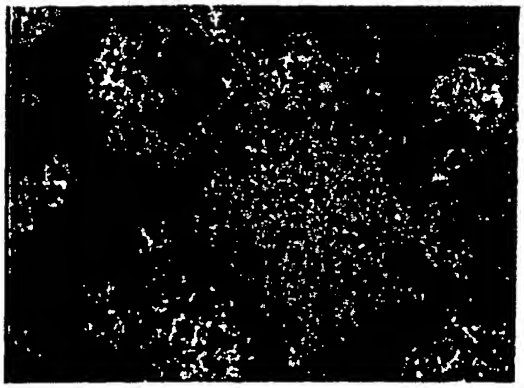
Figure 4



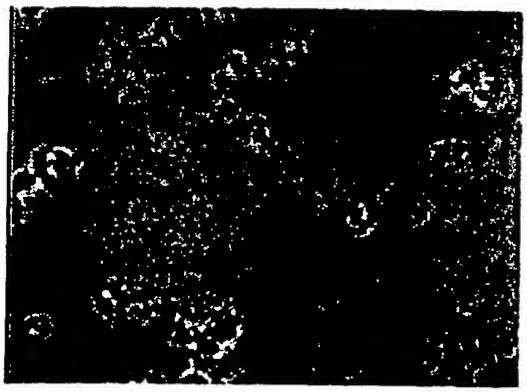
a



b



c



d

e

f

Figure 5

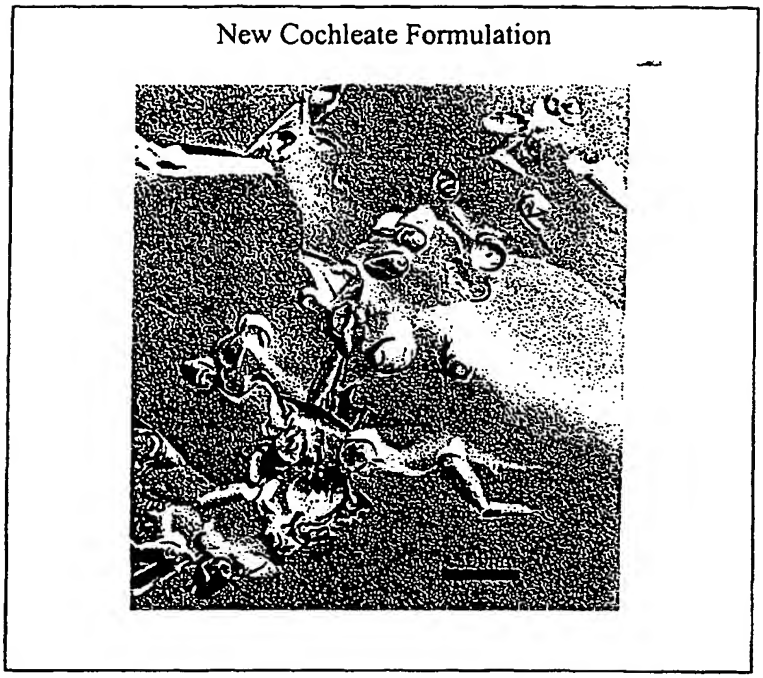


Figure 6

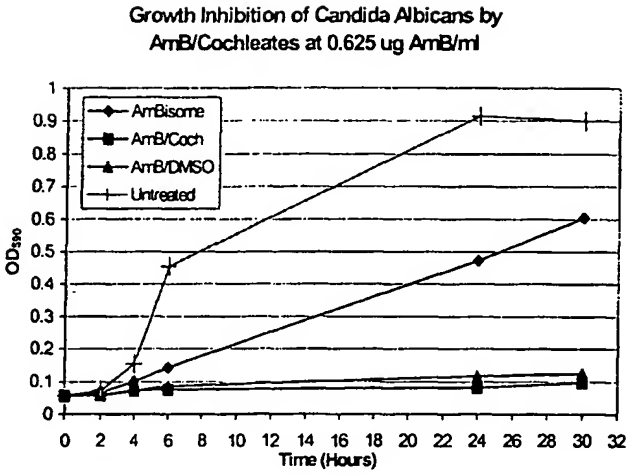

Growth Inhibition of Candida Albicans by AmB/Cochleates at 0.625 ug AmB/ml
AmBisome
AmB/Coch
AmB/DMSO
Untreated
OD590
Time (Hours)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32

Figure 7

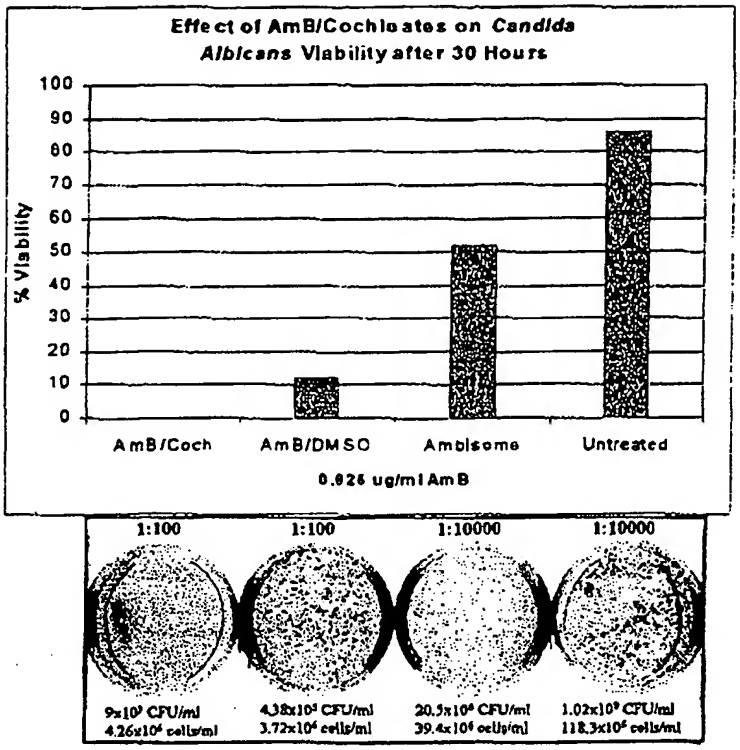
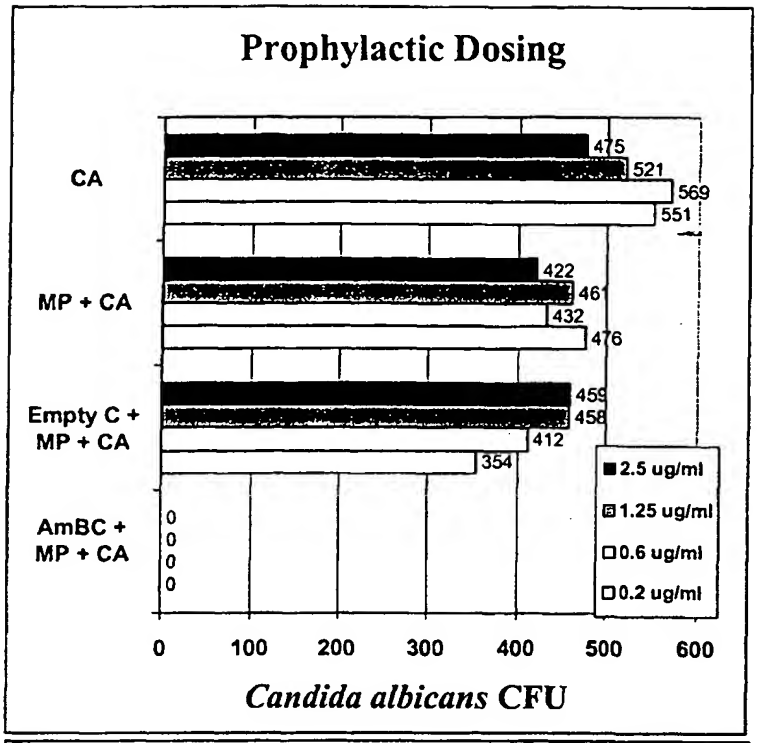

Effect of AmB/Cochleates on *Candida Albicans* Viability after 30 Hours
% Viability
100
90
80
70
60
50
40
30
20
10
0
AmB/Coch
AmB/DMSO
Ambisome
Untreated
0.025 ug/ml AmB
1:100
1:100
1:10000
1:10000
9x10³ CFU/ml
4.26x10⁶ cells/ml
4.38x10⁵ CFU/ml
3.72x10⁶ cells/ml
20.5x10⁶ CFU/ml
39.4x10⁶ cells/ml
1.02x10⁹ CFU/ml
118.3x10⁶ cells/ml

Figure 8


Prophylactic Dosing
CA
MP + CA
Empty C + MP + CA
AmBC + MP + CA
475
521
569
551
422
461
432
476
459
458
412
354
0
0
0
0
2.5 ug/ml
1.25 ug/ml
0.6 ug/ml
0.2 ug/ml
0
100
200
300
400
500
600
Candida albicans CFU

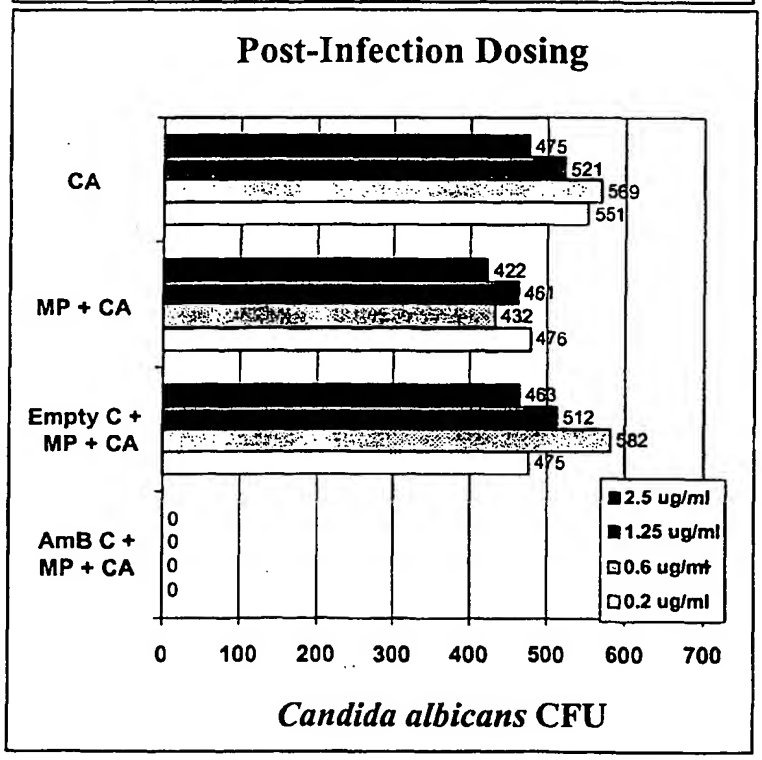

Post-Infection Dosing
CA
MP + CA
Empty C + MP + CA
AmB C + MP + CA
475
521
569
551
422
461
432
476
463
512
582
475
0
0
0
0
2.5 ug/ml
1.25 ug/ml
0.6 ug/ml
0.2 ug/ml
0
100
200
300
400
500
600
700
Candida albicans CFU

Figure 9. AmB tissue exposure based on the AUC

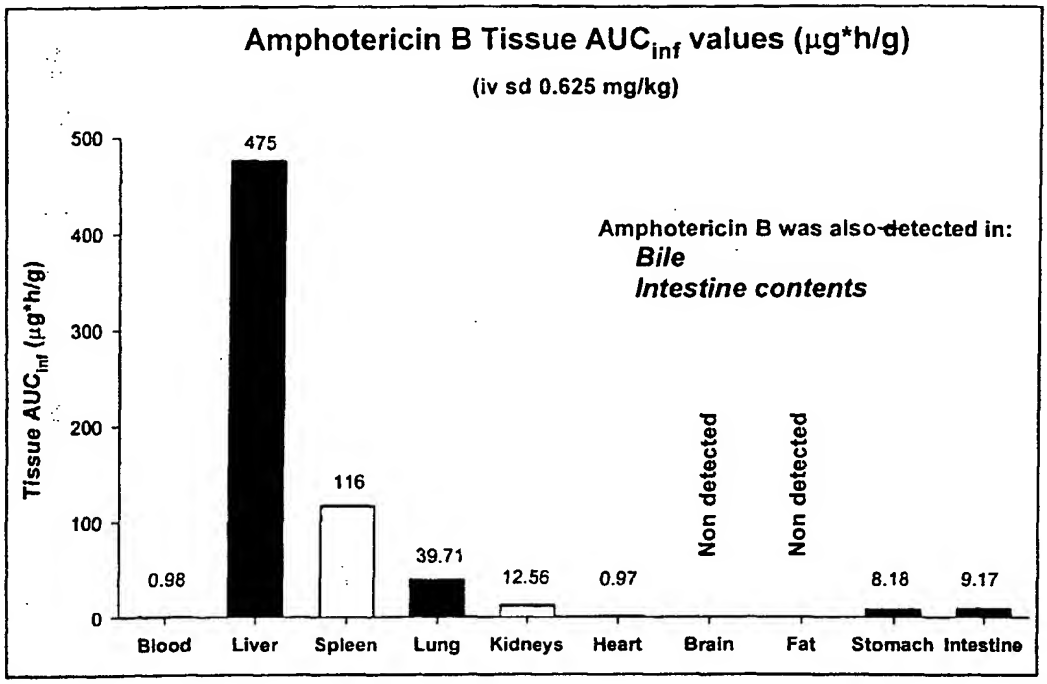


Figure 10

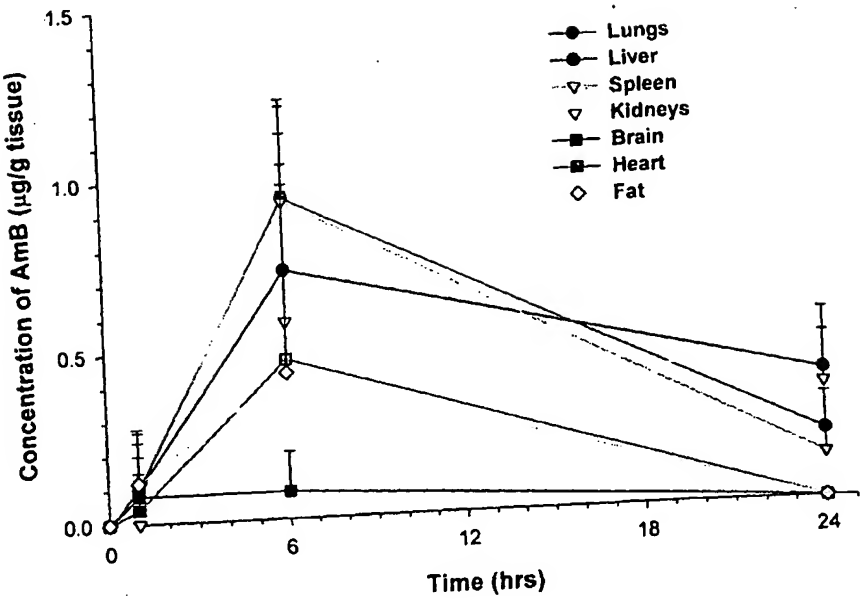

Concentration of AmB (µg/g tissue)
1.5
1.0
0.5
0.0
0
6
12
18
24
Time (hrs)
Lungs
Liver
Spleen
Kidneys
Brain
Heart
Fat

Figure 11

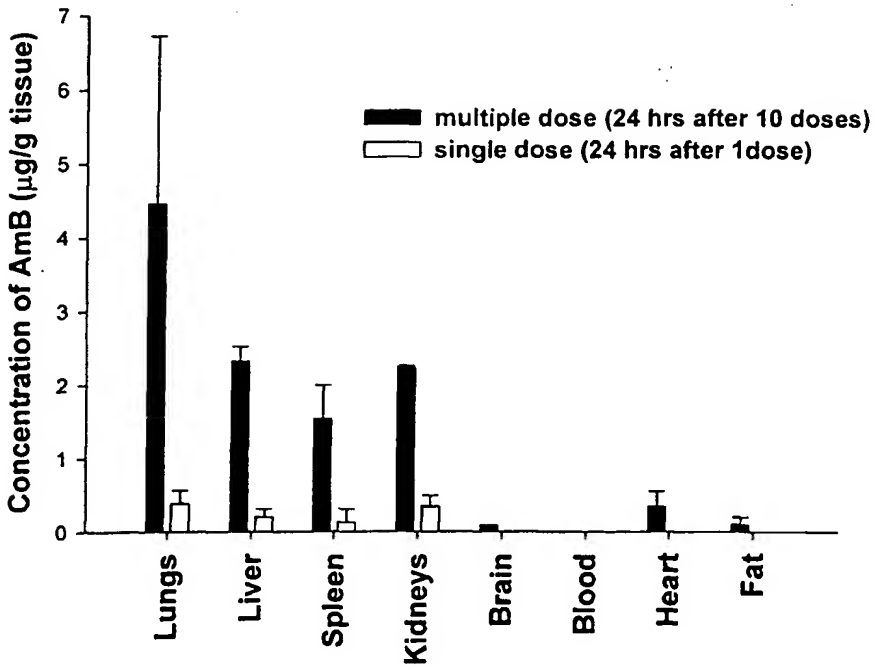
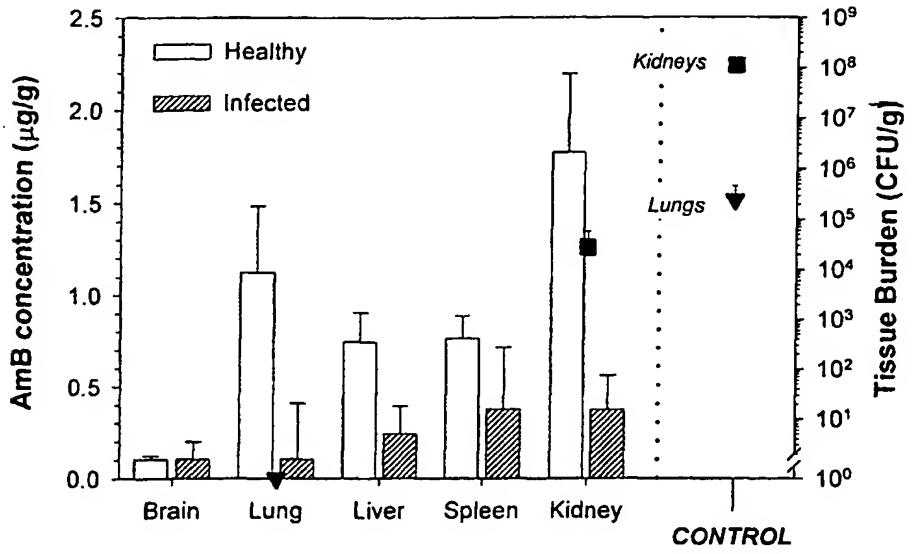

Concentration of AmB (µg/g tissue)
0
1
2
3
4
5
6
7
multiple dose (24 hrs after 10 doses)
single dose (24 hrs after 1dose)
Lungs
Liver
Spleen
Kidneys
Brain
Blood
Heart
Fat

Figure 12


AmB concentration (μg/g)
Tissue Burden (CFU/g)
Healthy
Infected
Kidneys
Lungs
2.5
2.0
1.5
1.0
0.5
0.0
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
Brain
Lung
Liver
Spleen
Kidney
CONTROL